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INTRAHIPPOCAMPAL CHOLINESTERASE INHIBITION INDUCES EPILEPTOGENESIS IN MICE WITHOUT EVIDENCE OF NEURODEGENERATIVE EVENTS

F. PERNOT,[a]* P. CARPENTIER,[a] V. BAILLE,[a] G. TESTYLIER,[a] C. BEAUP,[a] A. FOQUIN,[a] P. FILLIAT,[a] P. LISCIA,[b] M. COUTAN,[b] C. PIÉRARD,[b] D. BÉRACOCHEA[c] AND F. DORANDEU[a]

[a]*Département de Toxicologie, Centre de Recherches du Service de Santé des Armées, CRSSA, 24 Avenue des Maquis du Grésivaudan, B.P. 87, 38702 La Tronche cedex, France*

[b]*Département de Physiologie Intégrée, Institut de Médecine Aérospatiale du Service de Santé des Armées (IMASSA), B.P. 73, 91223 Brétigny sur Orge cedex, France*

[c]*Centre de Neurosciences Intégratives et Cognitives, Unité Mixte de Recherche 5228, Centre National de la Recherche Scientifique (CNRS), Universités de Bordeaux 1 et 2, Avenue des Facultés, 33405 Talence cedex, France*

Abstract—The mechanisms of epileptogenesis remain largely unknown and are probably diverse. The aim of this study was to investigate the role of focal cholinergic imbalance in epileptogenesis. To address this question, we monitored electroencephalogram (EEG) activity up to 12 weeks after the injection of a potent cholinesterase (ChE) inhibitor (soman) at different doses (0.53, 0.75, 1, 2, 2.8, 4 and 11 nmol) into the right dorsal hippocampus of C57BL/6 mice. Different parameters were used to choose the dose for a focal model of epileptogenesis (mainly electrographic patterns and peripheral ChE inhibition). The pattern of neuronal activation was studied by Fos immunohistochemistry (IHC). Brain damage was evaluated by hemalun-phloxin, neuronal nuclei antigen IHC and silver staining. Glial fibrillary acidic protein IHC was used to evaluate astroglial reaction. Finally, long-term behavioral consequences were characterized. At the highest dose (11 nmol), soman quickly evoked severe signs, including initial seizures and promoted epileptogenesis in the absence of tissue damage. With lower doses, late-onset seizures were evidenced, after 1–4 weeks depending on the dose, despite the absence of initial overt seizures and of brain damage. Only a weak astroglial reaction was observed. Following injection of 1 nmol, Fos changes were first evidenced in the ipsilateral hippocampus and then spread to extrahippocampal areas. A selective deficit in contextual fear conditioning was also evidenced two months after injection. Our data show that focal hypercholinergy may be a sufficient initial event to promote epilepsy and that major brain tissue changes (cellular damage, edema, neuroinflammation) are not necessary conditions.

Key words: epilepsy, acetylcholine, astrogliosis, Fos, fear conditioning, mouse.

Epilepsy afflicts 50 million people worldwide (Strine et al., 2005) and the temporal lobe epilepsy (TLE) is among the most frequent types of drug-resistant epilepsy (Acharya et al., 2008). TLE can be induced by an initial precipitating event (IPE) such as status epilepticus (SE), head trauma or other types of brain insults. There is usually a latent period of several years between this IPE and the emergence of epilepsy. The term of epileptogenesis is used to define the complex and ill-known modifications that take place during this period of time. Most of the anti-epileptic drugs currently used have little impact on epileptogenesis and merely provide symptomatic treatment. Identifying some of the key mechanisms would help to design more efficient drugs.

Acetylcholine (ACh) is a fundamental neurotransmitter in the central nervous system (CNS), where it is critically implicated in functions related to a variety of physiological processes (Decker and McGaugh, 1991) and is also involved in epileptic mechanisms. Indeed, a direct muscarinic stimulation with the agonist pilocarpine induces SE (Honchar et al., 1983; Turski et al., 1989). Seizures provoked by organophosphorus nerve agents like soman, which are potent irreversible inhibitors of cholinesterase (ChE), the enzyme that hydrolyses ACh, also have a cholinergic starter but the glutamatergic system is also rapidly recruited and responsible for the maintenance of seizures and excitotoxicity (Lallement et al., 1991). Moreover, specific mutations of ACh receptors have been described in an idiopathic epileptic syndrome and its animal models (Steinlein et al., 1995; Klaassen et al., 2006; Teper et al., 2007). A cholinergic implication in TLE was equally suggested (Friedman et al., 2007). Nevertheless, the epileptogenetic consequences of focal cholinergic changes in seizure-prone structures such as hippocampus remain little known. The focal injection of pilocarpine into rat hippocampus has been shown to produce an initial SE, significant neurochemical changes, including an increase in glutamate extracellular concentrations (Smolders et al., 2004) and spontaneous recurrent seizures (Furtado et al., 2002) over the observation period of 30 days. Conversely, the focal administration of soman in rat hippocampus failed to initi-

*Corresponding author. Tel: +33-0-4-76-63-68-43; fax: +33-0-4-76-63-69-62.
E-mail address: fabien.pernot@gmail.com (F. Pernot).

Abbreviations: ACh, acetylcholine; AMN, atropine methyl nitrate; ANOVA, analysis of variance; AUC, area under the curve; BSA, bovine serum albumin; ChE, cholinesterase; CNS, central nervous system; CS, conditioned stimulus; DAB, diaminobenzidine; DG, dentate gyrus; EEG, electroencephalogram; GFAP, glial fibrillary acidic protein; H&P, hemalun-phloxin; IHC, immunohistochemistry; IPE, initial precipitating event; KA, kainate; NeuN, neuronal nuclei antigen; SE, status epilepticus; TBE, time spent in the closed arm; TBO, time spent in the open arm; TBS, Tris-buffered saline; TLE, temporal lobe epilepsy; US, unconditioned stimulus; WB ChE, whole-blood cholinesterase.

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ate seizures (McDonough et al., 1987). Unfortunately animals were only observed for 6 h thus preventing the assessment of longer term effects such as epileptogenesis. Therefore, in order to gain more insights into the implication of cholinergic changes in the early steps of epileptogenesis, we studied over several weeks the consequences of the ChE inhibition provoked by the focal administration of soman in the hippocampus of C57BL/6 mice. Several aspects were studied: (i) acute and long-term electroencephalogram (EEG) changes; (ii) histopathology including neuronal damage and astroglial reaction; (iii) ChE inhibition; (iv) pattern of expression of the immediate early gene protein product, Fos, a surrogate marker of increased neuronal activity and (v) long-term behavioral consequences.

EXPERIMENTAL PROCEDURES

Animals

The experiments performed in the present study were approved by the Institutional Animal Care and Research Advisory of the Centre de Recherches du Service de Santé des Armées in accordance with the current and appropriate European and French legislation. All experiments conformed to international guidelines on the ethical use of animals and all efforts were made to minimize the number of animals used and their suffering. Male C57BL/6 mice (Janvier, Le-Genest-St-Isle, France) of 8–10 weeks of age were housed in groups of five to 10 for 7–14 days prior to surgery. They were given normal laboratory food and water *ad libitum* and kept in a 12-h light/dark cycle with light provided between 7 AM and 7 PM; after surgery, mice were similarly housed but in individual cages. All experiments were performed between 7:00 AM and 1:00 PM.

Preparation of soman solution

Soman (>97% pure by gas chromatography analysis) was supplied by the Centre d'Etudes du Bouchet (Vert-le-Petit, France). The injection volume was kept constant (50 nl) except for the highest dose (11 nmol), for which volume had to be increased to 100 nl owing to soman solubility in water. The different doses were obtained by changing the concentration of the solution that was extemporarily prepared in ice cold 0.9% sterile saline (1.9 to <20 mg/ml).

Surgical procedures

Drug injection. Mice were anesthetized with chloral hydrate (400 mg/kg i.p.) and stereotaxically injected with the appropriate solution of soman into the right (hereafter referred to as injected or ipsilateral) dorsal hippocampus (anteroposterior −2 mm; mediolateral −1.5 mm; dorsoventral −2 mm; with bregma as the reference; Franklin and Paxinos, 2001) using a stainless steel cannula (outer diameter, 0.28 mm) connected to a 0.5 μl microsyringe (Hamilton, Bonaduz, Switzerland) via a PE-20 tubing filled with purified water. Injections were performed over 1 min using a micropump (CMA/100; Carnegie Medicine, Stockholm, Sweden). At the end of the injection, the cannula was left in place for an additional 2 min to limit backflow along the cannula track. Different doses of soman were injected (0.53, 0.75, 1, 2, 2.8, 4 and 11 nmol). Unless specified, control mice were sham-injected animals that received 50–100 nl of saline under the same conditions to match the soman groups.

Another paradigm was also used in which a cannula and EEG electrodes (see below) were chronically implanted under chlorate anesthesia 7 days before the injection to study the immediate EEG changes (see Experimental design).

Implantation of EEG electrodes and monitoring of seizures. Immediately after injection, an hippocampal electrode consisting of two twisted wires (stainless steel, polyester insulated, 0.125 mm of diameter), which tips were vertically separated by 0.3–0.5 mm, was implanted in the hippocampus at the injection site, with the same coordinates as for the cannula (see above). Three cortical electrodes were implanted bilaterally in the frontal cortex and in the left parietal cortex. The reference electrode was placed over the vermis of the cerebellum. Electrodes were connected to a multipin socket and secured to the skull with acrylic dental cement. To detect seizures, the EEG signals were recorded (band-passed-filtered between 0.5 and 50 Hz; Reega Minidix TR, Alvar Electronic, Paris, France), and then digitized and processed by fast-Fourier transform at 200 Hz using an in-house LabVIEW-based (National Instruments, Austin, Tx, USA) analysis program. The EEG was also visually analyzed for characteristic paroxysmal activity.

Histochemistry and immunohistochemistry

Soman- and saline-injected mice were deeply anesthetized with an overdose of pentobarbital (80 mg/kg i.p.) at different times depending on the experiment (see the following section). Mice were transcardially perfused at a flow of 5 ml/min with heparinated saline (5000 UI/ml, 25 ml), then with acetic formaldehyde in 0.9% saline (4% final, 30 ml). The brains were removed from the skull and postfixed in saline formaldehyde (4% final) for 24 h at 4 °C before paraffin embedding. Brains were then cut in coronal sections of 7 μm on a microtome and mounted onto slides. Adjacent microtome sections were used to study neuronal damage/loss and edema as well as astroglial activation and proliferation: (i) the correct location of the injection site, the occurrence of neuronal damage/loss (acidophilic cells) and brain edema were visualized by hemalun-phloxin (H&P) staining using a standard protocol (Baille et al., 2005); (ii) neurons were specifically identified using neuronal nuclei antigen (NeuN) and astrogliosis was assessed by detecting glial fibrillary acidic protein (GFAP). Briefly, after rehydration, sections were incubated for 10 min at room temperature in Tris-buffered saline (TBS; 0.15 M NaCl, 0.1 M Tris–HCl, pH 7.5) containing 0.3% v/v H_2O_2. After saturation of unspecific sites in TBS-BSA (TBS containing 1% w/v bovine serum albumin, BSA; Sigma-Aldrich, Lyon, France) for 1 h at room temperature, the sections were incubated overnight at 4 °C with the primary antibody (mouse monoclonal anti-NeuN, 1:400, Chemicon, Temecula, CA, USA; rabbit polyclonal anti-GFAP, 1:1000, Dako, Glostrup, Denmark) in TBS-BSA. After three 15 min-washings in TBS, the sections were treated with the appropriate secondary antibody (NeuN, biotinylated goat anti-mouse IgG, 1:1000; GFAP, biotinylated goat anti-rabbit IgG, 1:500; Vector Laboratories, Burlingame, CA, USA) in TBS-BSA for 1 h at room temperature and, finally, labeling was revealed using Vectastain ABC Elite® kit (Vector Laboratories) and diaminobenzidine (DAB; fastTM DAB tablet set, Sigma-Aldrich).

Soman diffusion in CNS was estimated by ChE histochemistry on coronal and sagittal cryostat sections (14 μm), obtained from brain snap-frozen in isopentane (−40 °C) as described previously (Kobayashi et al., 1994). ChE inhibition was estimated by a zone of bleaching on sections separated by 250 μm.

Neuronal activation and neurodegeneration were studied, by Fos protein immunocytochemistry and silver staining respectively, on free-floating sections. After pentobarbital deep anesthesia, mice were perfused as already described except for the fixative that was paraformaldehyde (4% in 0.9% saline). After removal, brains were cryoprotected (30% sucrose, 48 h at 4 °C) and adjacent cryostat coronal sections (40 μm, −3 to −1 mm from bregma) were collected in phosphate-buffered saline 0.1 M, pH